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Concurrent impairment of $(Na^+ + K^+)$-ATPase activity in multi-organ of type-1 diabetic NOD mice

Qixin Shi [a], Junyi Zeng [a,b], Yugang Dong [b], Kai Y. Xu [a,*]

[a] Department of Surgery, University of Maryland School of Medicine, Baltimore, Maryland 21201, USA
[b] Department of Cardiology, First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, Guangdong 510080, China

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ABSTRACT

Background: Type-1 diabetes causes serious complications. Detailed molecular pathways of type-1 diabetes-mediated organ dysfunction are not completely understood. Significantly depressed $(Na^+ + K^+)$-ATPase (NKA) activity has been found in erythrocytes, pancreatic β-cells, nerve cells, and muscle tissues of type-1 diabetic patients and rodent animal models. The characteristics of NKA in the development of the type-1 diabetes-mediated complications remain obscure. Here we investigated whether alterations of NKA activity in heart, kidney, and pancreas of type-1 diabetic mice occur simultaneously and whether depressed NKA activity is a universal phenomenon in major organs in the development of type-1 diabetes-induced complications.
Methods: Female non-obese diabetic (NOD) and non-obese resistant mice were used for the study. Mice blood glucose was monitored and ouabain-sensitive NKA activity was determined.
Results: Experimental results reveal that reduced NKA activity correlates with the progression of elevated blood glucose along with marked altered NKA apparent Na^+ affinity in all three organs of NOD mice. No significant changes of NKA protein expression were detected while the enzyme activity was reduced in tested mice, suggesting an inactive form of NKA might present in different tissues of the NOD mice.
Conclusion: Our study suggests that concurrent impairment of NKA function in multi-organ may serve as one of the molecular pathways participating in and contributing to the mechanism of type-1 diabetes-induced complications in NOD mice. A successful protection of NKA function from injury might offer a good intervention for controlling the progression of the disease.

1. Introduction

Insulin-dependent type-1 diabetes (Gale, 2002; Mathis, Vence, & Benoist, 2001) induces devastating complications including heart failure, kidney failure, blindness, nerve damage and stroke (Brownlee, 2001; The Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Research Group, 2000; The Diabetes Control and Complications Trial Research Group, 1993). To date, detailed molecular pathways underlying the type-1 diabetes-mediated complications are not completely understood. Understanding the molecular processes and the mechanisms of type-1 diabetes-mediated complications will help to develop therapeutic approaches for preventing and treating the type-1 diabetes-mediated complications. $(Na^+ + K^+)$-ATPase (NKA), also called Na pump, is a key enzyme expressed in all animal cells (Kyte, 1981; Morth et al., 2007; Skou, 1988). It comprises two basic α and β subunits, and catalyzes the hydrolysis of ATP for the active transport of Na^+ and K^+ ions across the plasma membrane as required for cellular uptake of ions, glucose, amino acids, nutrients and neurotransmitters that are vital to cell function (Ewart & Klip, 1995; Geering, 2008; Jorgensen, Hakansson, & Karlish, 2003; Kyte, 1981; Lingrel & Kuntzweiler, 1994; Morth et al., 2007; Skou, 1988). Significantly depressed NKA activity has been found in erythrocytes (De La Tour et al., 1998), pancreatic β-cells (Owada et al., 1999), nerve cells (Greene, Lattimer, & Sima, 1987; Jannot, Raccah, De La Tour, Coste, & Vague, 2002; Sima & Sugimoto, 1999), cardiac and skeletal muscles (Kjeldsen, Braendgaard, Sidenius, Larsen, & Norgaard, 1987) of type-1 diabetic patients and rodent animal models. However, it is uncertain whether depressed NKA activity may have a universal biochemical effect in all organ systems during the development of type-1 diabetes. The nature of the involvement of NKA in the progression of the type-1 diabetes-mediated complications remains obscure. This study is designed to examine whether alterations of NKA activity and its Na^+ affinity occur simultaneously in heart, kidney, and pancreas of type-1 diabetic animal model, correlating with the progression of elevated blood glucose concentrations. The non-obese diabetic (NOD) mouse model (Atkinson & Leiter, 1999; Karounos & Goes, 2003) was chosen for the study since it is an excellent tool for investigators to evaluate the pathogenesis of type-1 diabetes-induced complications (Atkinson & Leiter, 1999; Karounos & Goes, 2003).

* Corresponding author. University of Maryland School of Medicine, Department of Surgery, MSTF-434E, 10 South Pine Street, Baltimore, Maryland, USA. Tel.: +1 410 706 2598; fax: +1 410 706 0311.
E-mail address: kxu002@umaryland.edu (K.Y. Xu).

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2. Materials and Methods

2.1. Materials

General chemicals were purchased from Sigma Chemical unless otherwise specified. Ouabain (>99%) was purchased from Fluka BioChemika (Buchs, Switzerland). Sodium azide (NaN_3) (Muneyuki et al., 1993) and thapsigargin (Tg) (Sagara & Inesi, 1991) were from Sigma-Aldrich (St. Louis, MO, USA). Western Blue and anti-rabbit IgG were from Promega (Madison, WI, USA). Semi-Dry Transfer Cell, sample buffer, running and transfer buffers were from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Rabbit polyclonal antibody SSA412 was generated against the H7-H8 domain of rat NKA (Cytomol, CA, USA) (Lee et al., 2009; Xu, 2005).

2.2. Animal models

Female non-obese diabetic mice (NOD/LtJ) and non-obese resistant (NOR/LtJ, as control) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) (Anderson & Bluestone, 2005; Prochazka, Serreze, Frankel, & Leiter, 1992). Animals were fed standard laboratory diet and maintained under specific pathogen-free conditions in the animal facility at the University of Maryland School of Medicine.

2.3. Monitoring mice blood glucose

Blood (1 μl) was obtained by pricking the tail of immobilized mice within the restraint device with a sterile 30 gauge needle. The 1 μl blood was then analyzed using an OneTouch hand held glucose analyzer. Direct pressure with gauze is applied following blood collection from the tail-vein. Mice in vivo blood glucose level was classified as normoglycemic (<11 mM), diabetic (≥15 mM) (Burke, Dong, Hazan, & Croy, 2007), and overt (≥33 mM).

2.4. Method of euthanasia and harvesting organ tissues

Mice were euthanized with sodium pentobarbital (≥100 mg/kg) and the different organs (heart, kidney, and pancreas) were removed immediately and rinsed in an ice-cold solution containing 10 mM histidine (free base) and 0.75 M NaCl, 0–4 °C. The Animal Care and Use Committees of the University of Maryland School of Medicine approved the protocol.

2.5. Sample preparation and Western blotting

In vivo blood glucose concentrations were determined prior to the isolation of organs from both NOR and NOD mice. Hearts, kidneys, and pancreases of mice were collected at different in vivo blood glucose concentrations including 16–20 and 33 mM. Harvested organs from NOR mice have normal blood glucose concentration around 5.5–6.6 mM and were used as controls throughout the study. Organs were rinsed in an ice-cold buffer solution containing 10 mM histidine (free base) and cut into 1 mm pieces and homogenized for 5 s at 14,000 rpm for three times. Following the determination of protein concentrations, samples (30 μg each) were mixed with electrophoresis sample buffer, boiled for 5 min, and loaded on a 7.5% SDS-gel. Following electrophoresis with a low voltage (70 V) for 120 min, the samples were transferred from the SDS gel to a nitrocellulose membrane (0.45 μm) for 30 min using a semi-dry electroblotting apparatus. The nitrocellulose membrane was blocked with 5% milk for 1 h and incubated overnight with SSA412 antibody (1:1000) separately. The membranes were then washed and incubated with alkaline phosphatase conjugated secondary antibody (1:7500) for 1 h. The color was developed using Western Blue for visual analysis.

2.6. NKA activity assay

The NKA activity of both NOR (as control) and NOD mice was measured based on the method of Kyte (Kyte, 1971) with modifications as described previously (Xu, 2005). The enzymatic activity is defined as the ouabain-sensitive hydrolysis of MgATP in the presence of Na^+ and K^+. Briefly, the incubation mixture contained 10 mM Tris/HCl, pH 7.4, 100 mM NaCl, 20 mM KCl, and tissue microsome sample with or without 2 mM ouabain in a final volume of 0.2 ml and was brought to and maintained at 37 °C in a water bath. The reaction was initiated by adding MgATP (3 mM) at 37 °C and stopped after 30 min by adding 0.75 ml quench solution (0.5% ammonium molybdate + 0.5 M H_2SO_4) and 0.02 ml developer (25 mg/ml of a mixture of 0.2 g 1-amino-2-naphthol-4-sulfonic acid + 1.2 g sodium bisulfate + 1.2 g sodium sulfite). The color was allowed to develop for 30 min at room temperature, and the phosphate generated in the reaction was then determined at 700 nm using a spectrophotometer (Xu, 2005). The NaN_3 (0.5 mM) and Tg (1 μM) were used during the assay to inhibit the activities of the F_0/F_1-ATPase and Ca^{2+}-ATPase (Muneyuki et al., 1993; Sagara & Inesi, 1991). To determine the apparent affinity of Na^+, various concentrations of Na^+ (0. 3, 5, 10, 20, 40, 80 and 100 mM) were applied with fixed K^+ (20 mM) and MgATP (3 mM). The isoforms of NKA may present in the heart, kidney, and pancreas organs tissues and all isoforms of NKA share the same catalytic function (Hayward et al., 1999; Lucchesi & Sweadner, 1991; Smith, Caplan, Forbush, & Jamieson, 1987). The NKA activity determined here represents a total NKA activity of each tissue sample. Alteration of a total NKA activity includes changed activity of NKA isoforms.

2.7. Statistics

All data were expressed as mean ± SEM. Student's paired *t* test was used to determine the statistical differences various experimental groups; $P<0.05$ was considered to be significant. SigmaPlot nonlinear regression was used to calculate the NKA apparent affinity ($K_{0.5}$) for Na^+.

3. Results

3.1. Concurrent reduction of the NKA activity is a function of in vivo elevated blood glucose concentration in multiple organs of NOD mice

NKA enzymatic activity of heart, kidney, and pancreas of NOD and NOR mice was determined under the same experimental conditions as described in the Methods. Samples from heart, kidney, and pancreas of NOR mice were used as controls throughout the study. When in vivo blood glucose concentration was increased from the normal level to 16–20 mM, ouabain-sensitive NKA activity of heart muscle, kidney, and pancreas was 75 ± 10%, 90 ± 11%, and 72 ± 8%, respectively, compared with the NKA activity of control NOR mice (Fig. 1A, B, & C). Experimental results show that NKA activity was further reduced to 43 ± 18%, 46 ± 9%, and 51 ± 13% for heart, kidney, and pancreas respectively when in vivo blood glucose was elevated to the level of 33 mM for all three organs of NOD mice (Fig. 1).

3.2. Impaired NKA activity is independent of protein expression

Having determined reduced NKA activity in all three organs of NOD mice, we next investigated whether significantly decreased NKA activity at the blood glucose concentration of 33 mM is due to a reduction of the enzyme molecular expression. The α-subunit of the enzyme contains the sites for binding of Na^+, K^+ and ATP, and is responsible for the catalytic activity of NKA. Therefore, Western